Biochemical and Biophysical Research Communications 394 (2010) 141–145

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mapping the CGRP receptor ligand binding domain: Tryptophan-84 of RAMP1 is critical for agonist and antagonist binding

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ARTICLE INFO

Article history:
Received 11 February 2010
Available online 24 February 2010

Keywords:
CGRP
Migraine
Telcagepant
GPCR

ABSTRACT

The calcitonin receptor-like receptor (CLR) associates with the accessory protein RAMP1 to form a receptor for the neuropeptide calcitonin gene-related peptide (CGRP). Multiple lines of evidence have implicated CGRP in the pathophysiology of migraine headache making the CGRP receptor an attractive target for development of small-molecule antagonists as a novel treatment for this debilitating condition. The CGRP receptor antagonists telcagepant and olcegepant (BIBN4096BS) have demonstrated clinical efficacy in the treatment of migraine and there is now a need to better understand how these molecules interact with the receptor. Previous work has shown the extracellular portion of RAMP1 to be important for binding of these antagonists, with tryptophan-74 being a key interaction site. The crystal structure of the extracellular portion of human RAMP1 placed tryptophan-74 in a hydrophobic patch hypothesized to interact with CGRP receptor ligands and also identified nearby residues that may be important for ligand binding. In this study we explored the role played by these residues of RAMP1 using an alanine replacement strategy. We confirmed a role for tryptophan-74 in antagonist binding and also identified arginine-67 as being important for binding of telcagepant but not compound 3, a close analog of BIBN4096BS. We also identified tryptophan-84 as being critical for both high-affinity binding of the non-peptide antagonists as well as the peptides CGRP and CGRP(8–37). These data for the first time pinpoint a specific RAMP1 residue important for both antagonist and agonist potency and are consistent with the N-terminal domain of RAMP1 forming the binding pocket interface with CLR.

1. Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid neuropeptide produced by alternative splicing of the calcitonin gene [1]. CGRP is widely expressed in the periphery and CNS and exhibits a range of biological functions with the most pronounced being vasodilation [2,3]. Multiple lines of evidence suggest CGRP plays a key role in the pathophysiology of migraine headache. Levels of CGRP in cranial circulation are increased during migraine [4] and treatment of migraine pain with a triptan normalizes CGRP levels [5]. It has also been shown that intravenous delivery of CGRP to migraineurs can lead to migraine-like headaches in some patients [6]. This body of evidence led to an effort to develop antagonists of the CGRP receptor as a new treatment for migraine. The first CGRP receptor antagonist to demonstrate clinical proof of concept was intravenously administered olcegepant [7]. More recently, the orally bioavailable small-molecule telcagepant has shown efficacy in phase II and phase III studies [8–10].

The success of these small-molecules in treating migraine in the clinic has led to interest in how these antagonists interact with the CGRP receptor. This is comprised of two membrane-spanning proteins, calcitonin receptor-like receptor (CLR), and receptor activity modifying protein 1 (RAMP1) [11]. CLR is a 7-transmembrane protein belonging to family B of G-protein coupled receptors (GPCRs) which are characterized by having peptide agonists which bind to large N-terminal extracellular domains [12]. CLR is retained intracellularly without association with one of three RAMP proteins, designated RAMP1, RAMP2, and RAMP3. In addition to allowing CLR to reach the cell surface, RAMPs also determine the pharmacology of the receptor. CLR association with RAMP1 forms the CGRP receptor while association with either RAMP2 or RAMP3 leads to a high-affinity adrenomedullin receptor [11]. Another family B GPCR, the calcitonin receptor (CTR), also can oligomerize with the RAMP proteins to form high-affinity amylin receptors [13].

Several non-peptide CGRP antagonists have been shown to exhibit significant species selectivity with higher affinity for the human than the rat CGRP receptor [14,15]. Studies with chimeric human/rat CGRP receptors have shown this species selectivity,

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doi:10.1016/j.bbrc.2010.02.131

and hence the high-affinity binding, is driven primarily by RAMP1. Furthermore, RAMP1 site-directed mutagenesis has revealed residue 74 in particular as playing a key role [14,16]. However, pharmacological studies on chimeric CLR/CTR receptors identified the region containing residues 37–63 of CLR to also be important for small-molecule antagonist binding, suggesting that both RAMP1 and CLR are involved in binding these compounds [17]. Building upon these observations, the methionine-42 of CLR as well as tryptophan-74 of RAMP1 have recently been shown to be important for binding both telcagepant and BIBN4096BS, consistent with the previous data [18].

The goal of the study presented here was to use site-directed mutagenesis to further explore residues of RAMP1 that may be involved in agonist and antagonist binding, based on the crystal structure of the N-terminus of RAMP1. We have replaced residues 67, 71, 74, 78, and 84 of the human RAMP1 protein with alanine and studied the effects of these mutations on the potency of the peptide agonist CGRP, the peptide antagonist CGRP(8–37), telcagepant, and a close analog of BIBN4096BS designated compound 3.

2. Materials and methods

2.1. Constructs

Human CLR and RAMP1 or myc-RAMP1 were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA) prior to mutagenesis. Site-directed mutagenesis was carried out by using the QuikChange Lightning kit from Stratagene (La Jolla, CA) according to the manufacturer's protocol. Transient transfection was used to express these constructs in HEK-293 cells.

2.2. Cell culture

HEK-293 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen) with 4.5 g/l glucose, 1 mM sodium pyruvate, and 2 mM glutamine supplemented with 10% heat inactivated fetal bovine serum, and 100 U/mL penicillin and 100 μg/mL streptomycin and maintained in incubators at 37 °C with 5% CO_2. HEK-293 cells were transiently transfected using Lipofectamine 2000 (Invitrogen) or polyethylenimine (for ELISA) as previously described [19]. For cAMP assays, cells were plated in tissue culture treated 10 cm dishes (Becton Dickinson, Franklin Lakes, NJ) 24 h prior to transfection. For the transfection, 4 μg of CLR and 4 μg RAMP1 were combined with 0.5 mL Opti-MEM (Invitrogen) in a 15 mL conical tube while 23 μL Lipofectamine 2000 was combined with 0.5 mL Opti-MEM in a separate tube. Both mixtures were incubated at room temperature for 5 min and then they were mixed together and incubated at room temperature for and additional 30 min. The transfection mix was then added dropwise to the cells and the dish was placed back in the incubator for 48 h.

2.3. cAMP assays

Accumulation of cAMP was determined using the CisBio HTRF cAMP dynamic assay kit (CisBio, Bedford, MA). HEK-293 cells transiently expressing CLR and RAMP1 were dissociated, spun down, and resuspended in assay buffer consisting of Cellgro COMPLETE Media (Mediatech, Manassas, VA) with 300 μM IBMX (Sigma, St. Louis, MO). Antagonist titrations were made in DMSO then transferred to a dry 384 well, opaque, white assay plate (Proxiplate plus, Perkin-Elmer, Waltham, MA). For agonist assays, DMSO was added to the plate. Cells were then added to the plate at a density of 2000 cells/well and the plate was incubated at room temperature for 30 min. For antagonist assays, an EC_{50} concentration of the peptide agonist α-CGRP was then added and the plate was incubated for 20 min at room temperature. For agonist assays, titrations of peptide agonist in assay buffer were added. Finally, the d2 and cryptate HTRF reagents were diluted in lysis buffer and added and the plate was incubated for 1 h at room temperature. The plate was then read using an Envision (Perkin-Elmer) plate reader in HTRF mode. The raw data were converted to nM cAMP using a standard curve.

2.4. Membrane preparation and radioligand binding assays

Transiently transfected HEK-293 cells were washed with ice-cold PBS then harvested in harvest buffer containing 50 mM HEPES, 1 mM EDTA, and Complete protease inhibitors (Roche, Indianapolis, IN). The cells were disrupted in a laboratory homogenizer and centrifuged at 48,000*g* for 30 min. The pellets were resuspended in harvest buffer with 250 mM sucrose and stored in aliquots at minus 70 °C. For binding assays, various concentrations of telcagepant and 10 μg membrane were incubated for 3 h in binding buffer (10 mM HEPES, 5 mM $MgCl_2$, and 0.2% BSA) containing 10 pM ^{125}I-CGRP (Perkin-Elmer). The binding reactions were terminated by filtration through polyethylene imine (0.5%) treated GF/B glass fiber filters using a cell harvester. Radioactivity was determined using a Topcount scintillation counter (Perkin-Elmer).

2.5. Cell surface expression of mutants by ELISA

Cell surface expression of myc-tagged mutant receptor RAMP1 was measured as previously described [19].

3. Results and discussion

The CGRP receptor is atypical in that it consists of a hetero-oligomer of the seven-transmembrane GPCR, CLR and the type I single transmembrane protein RAMP1. Previous studies using human/rat chimeric receptors have shown that RAMP1 is responsible for species selectivity of several small-molecule CGRP receptor antagonists suggesting that these molecules interact directly with this protein [14]. The crystal structure of the extracellular portion of human RAMP1 reveals a three-helical structure with a hydrophobic core with regions of the α2 helix thought to be important for antagonist binding [20]. The tryptophan at position 74 has been shown to facilitate binding of the CGRP receptor antagonists telcagepant and BIBN4096BS [18] and there are several other residues in proximity to W74 with side-chains exposed to the solvent which could also be potential sites of interaction for these antagonists or CGRP itself. Residues R67, D71, and E78 all are located on the α2 helix with W74 while W84 is located on the loop between α2 and α3 with its side-chain oriented in the same direction as W74 [20]. This information led us to explore whether these residues are important for the action of selected agonists and antagonists of the CGRP receptor.

Site-directed mutagenesis was carried out on 5 residues of human RAMP1 creating the mutants R67A, D71A, W74A, E78A, and W84A. In addition to these individual mutations, several double mutants were also generated including R67A + D71A, R67A + W74A, R67A + W84A, D71A + W74A, and W74A + W84A. Functional cAMP assays were conducted to determine the effect these mutations had on the potency of the agonist CGRP, the peptide antagonist CGRP(8–37), and the small-molecule antagonists telcagepant and compound 3 (Fig. 1). In addition, confirmatory radioligand competition binding studies were performed with telcagepant on selected mutants.

The potency of CGRP was determined for CGRP receptors consisting of human CLR and either wild type or mutant RAMP1 using a cAMP functional assay. The amount of cAMP produced by each concentration of CGRP was normalized to the amount of cAMP pro-

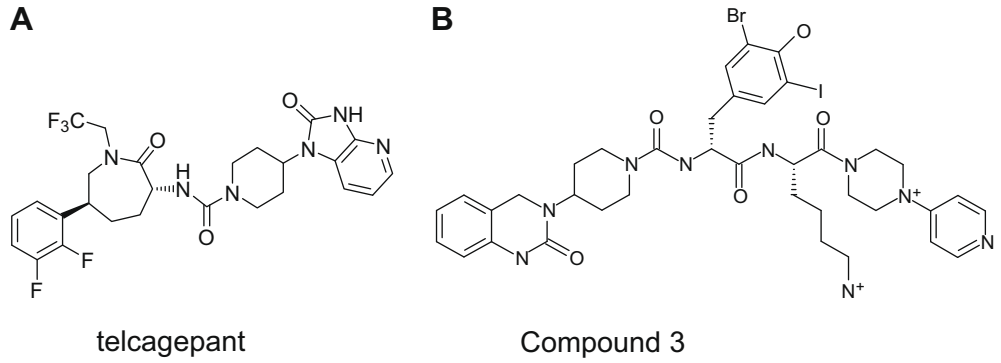

Fig. 1. Chemical structures of the CGRP receptor antagonists (A) telcagepant and (B) compound 3, an analog of BIBN4096BS.

duced by a saturating concentration of forskolin to determine if the RAMP1 mutations affected the efficacy of the agonist. The data from these experiments is summarized in Table 1 and represents the mean and SEM from at least three independent assays. The EC_{50} of CGRP was similar for all mutants tested with the exception of those containing the W84A mutation where the agonist was 20–30-fold less potent than on the wild type receptor (Table 1). The addition of either R67A or W74A to the W84A mutant did not have any additional affect on the potency of CGRP. None of the mutants tested caused a significant change in the efficacy of CGRP relative to forskolin.

The effects of these RAMP1 mutations on the potency of the CGRP receptor antagonists telcagepant, compound 3, and CGRP(8–37) were also tested in cAMP assays and the results are summarized in Table 2. The W84A mutation alone caused a decrease in potency for all three antagonists tested with an 18-fold reduction for telcagepant and a 7-fold reduction for both compound 3 and CGRP(8–37). The W74A mutation alone caused a decrease in potency for both telcagepant and compound 3 which is consistent with previously published results [18]. The combination W74A + W84A mutant showed a further reduction in potency for telcagepant and compound 3 to a level greater than either of these mutants alone. However, W74A alone had no effect on the potency of the peptide antagonist CGRP(8–37) and the combination W74A + W84A showed similar results to W84A alone. The R67A mutation had no effect on either compound 3 or CGRP(8–37) but it did cause a modest decrease in potency for telcagepant (~4-fold, Table 2). When R67A was combined with either the W74A or W84A mutations there was an additive effect for telcagepant with a 224- and 97-fold decrease in potency, respectively. Mutations of residues 71 and 78 did not affect any of the antagonists tested.

In addition to the cAMP assay, radioligand binding was also performed using telcagepant to test its affinity on CGRP receptors with wild type or mutant RAMP1. The R67A mutation reduced the affinity of telcagepant by 5-fold while the W74A mutation caused a 25-fold reduction in affinity (Fig. 2). The combination of R67A and W74A in a double mutant appeared to have an additive effect causing over 100-fold reduction in affinity. Alanine mutations at positions 71 and 78 had no effect on the IC_{50} of telcagepant consistent with functional data. The binding data shows a similar pattern as the functional data for the mutants we tested and further supports a role for both R67 and W74 for telcagepant binding. Unfortunately, we were unable to determine the affinity of telcagepant against the W84A mutant because the reduced affinity of [^{125}I]-CGRP for receptors with this mutation prevented us from obtaining a K_D with the commercially available radioligand.

ELISA assays were used to detect myc-tagged RAMP1 in order to determine if any of these mutations affected cell surface expression of the CGRP receptor. Wild type RAMP1 alone did not have sig-

Table 2
Antagonist potency in a cAMP functional assay.

	Telcagepant		Compound 3		CGRP(8–37)	
	pIC_{50}	±	pIC_{50}	±	pIC_{50}	±
hCLR/hRAMP1	9.30	0.03	9.87	0.04	8.10	0.03
hCLR/R67A	8.74	0.04	9.84	0.05	8.23	0.09
hCLR/D71A	9.22	0.02	9.72	0.04	8.01	0.03
hCLR/W74A	7.28	0.05	9.31	0.04	8.35	0.05
hCLR/E78A	9.56	0.03	9.83	0.02	8.19	0.04
hCLR/W84A	8.04	0.05	8.97	0.07	7.24	0.05
hCLR/R67A + D71A	8.55	0.03	9.65	0.03	8.27	0.04
hCLR/R67A + W74A	6.95	0.02	9.25	0.04	8.15	0.03
hCLR/R67A + W84A	7.30	0.04	8.96	0.03	7.13	0.04
hCLR/D71A + W74A	7.30	0.17	9.20	0.02	8.21	0.06
hCLR/W74A + W84A	6.19	0.06	7.75	0.04	7.40	0.08

Values represent mean and SEM of at least three independent experiments.

Table 1
α-CGRP potency in a cAMP functional assay.

	pEC_{50}	±	% Max Forskolin
hCLR/hRAMP1	9.66	0.04	73
hCLR/R67A	9.66	0.03	73
hCLR/D71A	9.74	0.03	74
hCLR/W74A	9.52	0.02	74
hCLR/E78A	9.85	0.06	76
hCLR/W84A	8.13	0.04	77
hCLR/R67A + D71A	10.06	0.03	82
hCLR/R67A + W74A	9.83	0.04	68
hCLR/R67A + W84A	8.33	0.03	82
hCLR/D71A + W74A	10.13	0.04	76
hCLR/W74A + W84A	8.37	0.03	80

Values represent mean and SEM of at least three independent experiments.

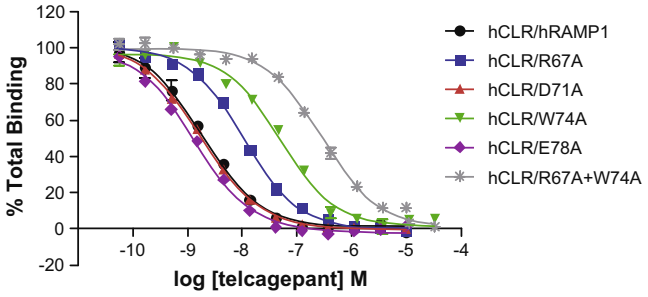

Fig. 2. Telcagepant competition binding. The ability of telcagepant to displace 10 pM [^{125}I]CGRP was tested on HEK-293 membranes expressing CGRP receptors with wild type and mutant RAMP1. The points represent the means ± SEM of combined data from at least three separate experiments.

nificant cell surface expression indicating that there is no significant endogenous expression of CLR. The myc-RAMP1 containing the W84A mutation was the only mutant to show significant reduction with 66% expression compared to wild type RAMP1 (Fig. 3). This reduction in cell surface expression is probably not sufficient to account for the degree of loss of agonist/antagonist potency as we have previously observed greater reductions in cell surface expression in RAMP1 mutants, which were not accompanied by substantial reductions in potency [27]. Further, CGRP showed similar efficacy to wild type RAMP1 in agonist induced cAMP accumulation relative to forskolin (Table 1).

The data presented here further confirms the important role that W74 plays in binding of small-molecule CGRP receptor antagonists. We also show that this residue does not appear to be important for the binding of the peptide antagonist CGRP(8–37), consistent with previous work showing that mutations at this position do not affect CGRP potency [16,21]. This supports the hypothesis that small-molecule antagonists and the peptide ligands of the CGRP receptor interact with different residues to achieve high-affinity binding. We have also identified residue R67 as playing a possible role in telcagepant binding to the CGRP receptor as there was a reduction in potency when this residue was mutated to alanine. Furthermore, there was a greater reduction in potency for telcagepant with the R67A + W74A double mutation than with either mutation alone. The same pattern was observed in both a functional cAMP assay as well as a radioligand binding assay. There was no observed reduction in either agonist potency or surface expression of the receptor which shows that this mutation does not interfere with the association of CLR with RAMP1. Interestingly, the R67A mutation had no effect on the potency of compound 3 either alone or in combination with W74A suggesting these two small-molecule antagonists may not have completely overlapping binding sites.

There has been discussion centered on whether small-molecule antagonists of the CGRP receptor such as telcagepant and BIBN4096BS are allosteric or competitive inhibitors. On one hand, Schild analysis using either telcagepant or BIBN4096BS show parallel rightward shifts of CGRP curves with no reduction in maximum effect over a wide range of antagonist concentrations, behavior that is indicative of competitive antagonism [22–24]. However, cold CGRP is unable to completely displace a tritiated version of telcagepant [25] and BIBN4096BS has been shown to accelerate the dissociation of [^{125}I]-Amylin from the AMY$_{1(a)}$ receptor which is consistent with an allosteric mechanism [26]. Also, none of the residues of RAMP1 or CLR that have been shown to affect small-molecule antagonist potency have an effect on the potency of the peptide agonist CGRP. In this report we show that mutation of W84 has a significant effect on the potency of the peptides CGRP and CGRP(8–37) as well as the non-peptide antagonists telcagepant and compound 3. One possible interpretation of these results is that all of these ligands interact directly with this residue. The side-chains of W84 and W74 are in close proximity and both are solvent exposed [20] so it makes sense that molecules like telcagepant that have been shown to interact with W74 could also interact with W84. This is further supported by the X-ray structure of the soluble ectodomain complex of CLR/RAMP1 bound to olcegepant which shows that this ligand interacts with the indole rings of both W74 and W84 [27]. The mutation of both of these residues to alanine in a double mutant leads to a reduction in potency for telcagepant and compound 3 that is significantly greater than either individual mutant. This interpretation is complicated by the observation that the W84A mutation leads to a modest loss in cell surface expression of the receptor. This makes it difficult to determine if the loss in potency of the peptides is due to direct interaction with this residue or a change in overall structure of the receptor resulting from disruption of the CLR/RAMP1 interface. However, other mutations have been shown to cause a larger decrease in cell surface expression without affecting CGRP potency [28]. W84 is in the loop between helix 2 and helix 3 while the proposed CLR interaction site is further down helix 3 [20]. Interestingly, the efficacy of CGRP (normalized to forskolin-induced cAMP accumulation) is the same on the W84A mutant as it is on the wild type receptor even though the potency is decreased. These data suggest the possibility that W84 is a common site of interaction for both the peptide ligands as well as small-molecule antagonists like telcagepant and compound 3. This common interaction site could explain why these antagonists appear to be competitive inhibitors in functional assays.

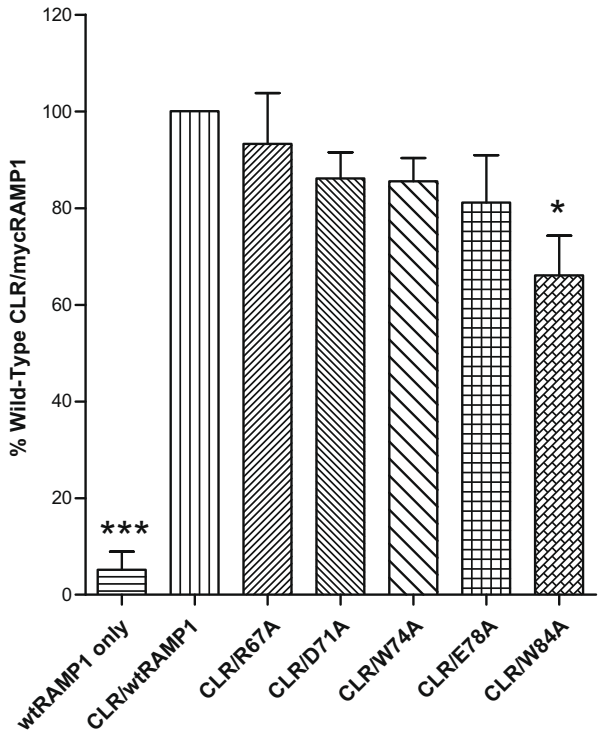

Fig. 3. Cell surface expression of myc-tagged wild type and mutant hRAMP1 determined by ELISA. $N = 3$ experiments with four replicates each. *, $P < 0.05$; ***, $P < 0.001$ by one-way ANOVA followed by Dunnett's test versus control.

4. Conclusion

In summary, we have identified residue R67 as being an interaction site for the small-molecule CGRP receptor antagonist telcagepant and confirmed residue W74 as being important for binding both telcagepant and the BIBN4096BS analog compound 3. We have also identified residue W84 as being important for potency of both peptides and small molecules and a possible common interaction site for the agonists and antagonists of the CGRP receptor. These data for the first time pinpoint a specific RAMP1 residue important for both antagonist and agonist potency and are consistent with the N-terminal domain of RAMP1 forming the binding pocket interface with CLR.

Acknowledgments

We thank Richard J. Bailey at the University of Auckland for performing the ELISA assays for myc-tagged RAMP1. We would also like to thank Anthony W. Shaw, Daniel V. Paone, and Ian M. Bell in the Department of Medicinal Chemistry at Merck Research Laboratories for synthesizing telcagepant and compound 3.

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